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Comparative Studies on the Interaction of Human and Bovine Serum Albumins with Vitamin C

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Abstract: The interactions of human and bovine serum albumins (HSA and BSA) with various drugs using spectroscopic techniques have received great attention now a days due to their significant effect in the biomedical field. UV absorption and fluorescence spectroscopy are the most likable due to its high sensitivity and simplicity. The interaction of HSA and BSA with vitamin C was investigated. Results showed that the absorption and fluorescence intensities increased as the vitamin C concentration increases. The calculated binding constant (k ~10^4 M^{-1}) showed a week binding of vitamin C with both serum albumins. The analysis of fluorescence quenching for HSA/BSA-vitamin C interaction (k_q ~ 10^{11} L mol^{-1} s^{-1}) reveals the dynamic quenching process and clearly confirms the existence of static mechanism of fluorescence quenching.

Keywords: Vitamin C, Human Serum Albumin, Bovine Serum Albumin, UV-Absorption, Fluorescence Spectroscopy, Binding Constant, Binding Mode

1. Introduction

Protein is an important chemical substance in our life and one of the main targets of all medicines in organism. Serum albumins, especially bovine (BSA) and human (HSA), labeled with fluorescent probes are commonly used for the investigation of surface induced conformational changes in protein interfaces [1-3]. From a biopharmaceutical point of view, one of the most important biological functions of albumins is their ability to carry drugs, endogenous and exogenous substances [4]. Both BSA and HSA have very high conformational adaptability to a great variety of ligands [5]. Some groups have studied the in vivo consequences of binding of drugs and other metabolites to serum albumins [6-8]. Others have examined the binding mechanism using absorption, fluorescence, circular dichroism etc [9-12]. Based on such studies, information on the binding process of many exogenous ligands like long chain fatty acids, amino acids, metals, drugs etc have been reported at the molecular level [13]. HSA and BSA, two of the most extensively studied serum albumins. However, there are still some differences between them [14]. HSA contains a single tryptophan (Trp-214) [15], while BSA has two tryptophan residues that possess intrinsic fluorescence: Trp-212 is located within a hydrophobic binding pocket of the protein and Trp-134 is located on the surface of the molecule [16]. Therefore, the experimental results of the interaction between drugs and BSA cannot be completely identical with those of HSA.

Therefore, study of the interaction between protein and drug molecules will help provide basic information on the pharmacological actions, bio-transformation, bio-distribution of drugs [17]. The binding phenomena will also be useful to explain the relationship between the structures and functions of proteins. There are some popular techniques which have been used to investigate the interaction between drugs and proteins. UV-absorption and fluorescence spectroscopy are of the powerful techniques to study molecular interactions which changes local environment of fluorophore and helps to predict the binding phenomenon of drugs to proteins [18-21]. There are a number of reports in the literature, where binding of metabolites, drugs, dyes, fatty acids, bio-active-substances have been studied in detail. So the nature of binding of a ligand with HSA (BSA) is different for different ligands [22 - 25].

Vitamin C or L-ascorbic acid, is an essential nutrient for

humans and certain other animal species. Vitamin C refers to a number of vitamins that have vitamin C activity in animals, including ascorbic acid and its salts, and some oxidized forms of the molecule like dehydroascorbic acid [26]. Vitamin C is a cofactor in at least eight enzymatic reactions, including several collagen synthesis reactions that, when dysfunctional, cause the most severe symptoms of scurvy [27]. In animals, these reactions are especially important in wound-healing and in preventing bleeding from capillaries [28]. Ascorbate may also act as an antioxidant against oxidative stress. Designated chemically as 2-Oxo-L-threo-hexono-1,4-lactone-2,3-enediol. The molecular weight is 176.12 g/mol. Its molecular formula is $C_6H_8O_6$ [29]. The structural formula is given in Figure 1:

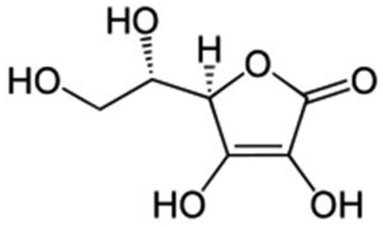


Figure 1. *The structure of L-ascorbic acid.*

In this work, this paper mainly studied about the interaction of human and bovine serum albumins with vitamin C using UV-absorption and fluorescence spectroscopic techniques, and to do a comparison between the binding behaviors of both proteins with vitamin C.

2. Experimental

2.1. Chemicals

HSA and BSA (purity > 99%) was purchased from Sigma Aldrich Company, and vitamin C was purchased from Al-Quds Co. pharmaceutical chemical industries, their solutions were prepared in phosphate buffer solutions (pH=7.4). The other substances are of reagent grade, and were used without further purifications.

A final concentration of (40 mg/ml) of HSA and BSA was used in the final vitamin C-protein solution, then the solution was placed in ultrasonic water path (SIBATA AU-3T) for one hour to ensure that all the amount of vitamin C was completely dissolved. The final concentrations of Vitamin C-Protein complexes were prepared by mixing equal volumes of HSA and BSA to equal volume from different concentration of vitamins C. HSA and BSA concentrations in all samples kept at 40mg.ml^{-1}. The final concentrations of the vitamin C are (40, 20, 10, 5, 2 and 1mg.ml^{-1}). The solution were incubated for 1 h (at 25°C) before spectroscopic measurements were taken.

2.2. Apparatus

UV-VIS spectrophotometer

The absorption spectra were obtained by the use of a NanoDrop ND-1000 spectrophotometer. It is used to measure the absorption spectrum of the samples in the range between 220-750 nm, with high accuracy and reproducibility.

Fluorescence spectrometer

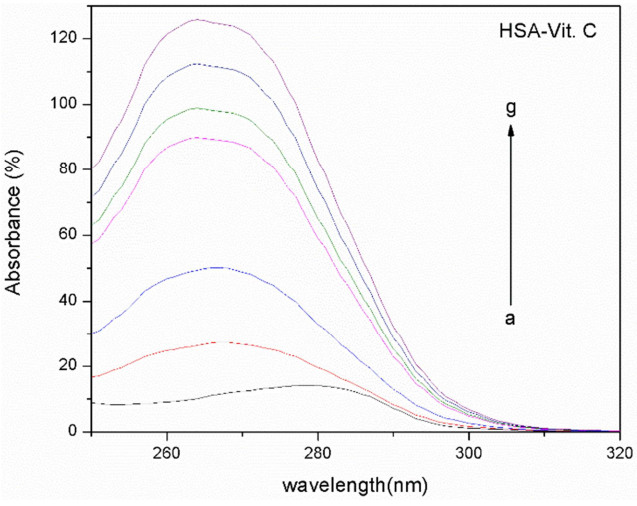
The fluorescence measurements were performed by a NanoDrop ND-3300 Fluoro-spectrophotometer at 25°C. The excitation source comes from one of three solid-state light emitting diodes (LEDs). The excitation source options include: UV LED with maximum excitation 365 nm, Blue LED with excitation 470 nm, and white LED from 500 to 650nm excitation. A 2048-element CCD array detector covering 400-750nm, is connected by an optical fiber to the optical measurement surface. The excitation is done at the wavelength of 360 nm and the maximum emission wavelength is at 439 nm.

The absorption and emission spectra were recorded for free HSA and BSA at 40mg.ml^{-1} and for its complexes with vitamin C solution with the concentrations of (40, 20, 10, 5, 2 and 1mg.ml^{-1}). Repeated measurements were done for all samples.

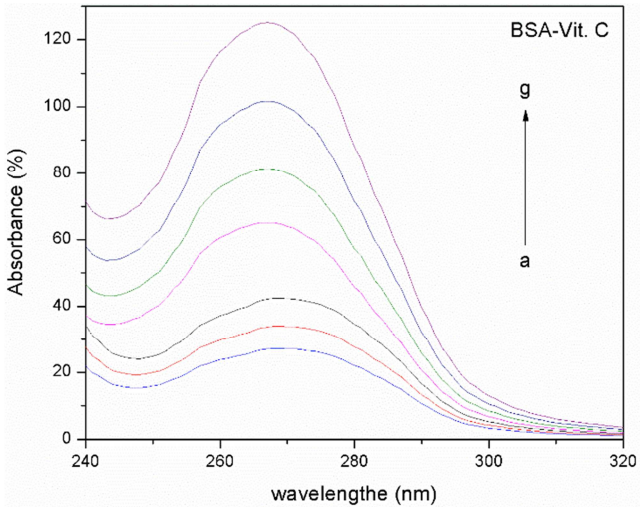
3. Results and Discussions

3.1. UV Absorption Spectra

UV–vis absorption measurement is a simple and pertinent method that is used to investigate structural changes and to explore complex formation [30]. To initially verify the quenching mechanism, the UV absorption spectra of HSA-vitamin C and BSA-vitamin C were measured and recorded. As shown in Figure 2, both proteins showed an absorption peak of 268 nm. With the addition of vitamin C, the intensity increased, indicating disturbances to the microenvironment around the amide bonds in the protein. The results demonstrated the existence of an interaction between HSA and BSA with vitamin C, this is due to major ligand protein interaction at protein surface which does not limit the mobility of ligand around protein molecule.



(a)



(b)

Figure 2. *UV-absorbance spectra of different concentrations of vitamin C (a=free HSA, b=1mg.ml^{-1}, c=2mg.ml^{-1}, d=5mg.ml^{-1}, e=10mg.ml^{-1}, f=20mg.ml^{-1}, g=40 mg.ml^{-1}) with a. HSA, b. BSA.*

The absorption data were treated using linear reciprocal plots based on the following equation [31].

$$\frac{1}{A-A_0} = \frac{1}{A_\infty - A_0} + \frac{1}{K[A_\infty - A_0]} \cdot \frac{1}{L} \quad (1)$$

where A_0 corresponds to the initial absorption of protein at 280 nm in the absence of ligand, A_∞ is the final absorption of the ligated protein, and A is the recorded absorption at different vitamin C concentrations (L). Figure 3 represents the double reciprocal plots of 1/(A- A_0) vs. 1/L for HSA-vitamin C and BSA-vitamin C complexes. The binding constant (K) can be estimated from the ratio of the intercept to the slope. The obtained values of the binding constants indicates a relatively weak interaction of vitamin C with HSA (K=1.28 x 10^4 M^{-1}) and with BSA (K= 1.39x10^4 M^{-1}) when compared to other drug-protein complexes with binding constants in the range of 10^5 and 10^6 M^{-1} [32-34]. The reason for the low stability can be attributed to the presence of mainly hydrogen-bonding interaction or an indirect Protein-vitamin interaction through water molecules [25].

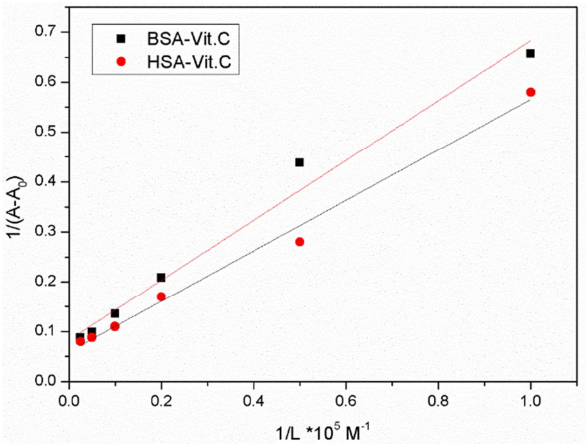
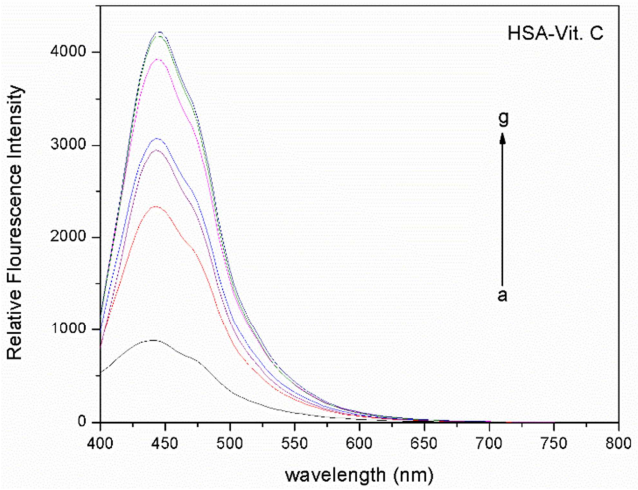


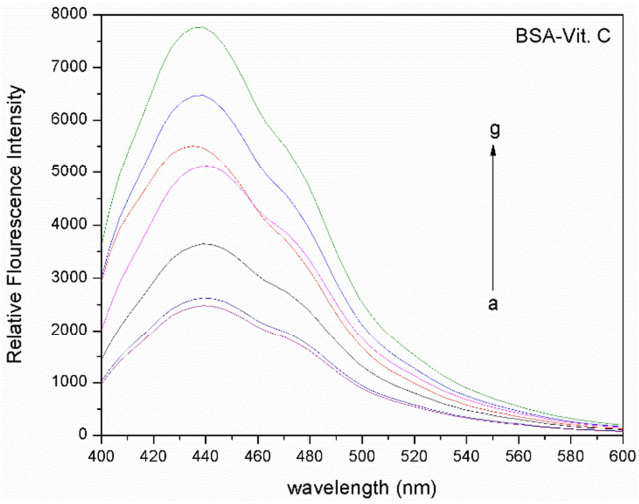
Figure 3. *The plot of 1/(A-A_o) vs. 1/L for HSA and BSA with different concentrations of vitamin C.*

3.2. Fluorescence Quenching

Fluorescence quenching studies to explore the binding interaction of drug ligands with proteins is considered as the best methodology [35]. This quenching resulted from the reduction of quantum yield of fluorescence from a fluorophore induced by a variety of molecular interaction with a quencher molecule [36, 37]. An Increase in the concentration of quencher will mask the fluorophore, result in reduction of emitted fluorescence from the fluorophore molecules. In ease of targeting specific location regarding the binding pattern of protein, we used different wavelength. Generally, the fluorescence of HSA and BSA comes from tryptophan, tyrosine and phenylalanine residues. During data recording at an excitation wavelength at 280 nm, fluorescence of albumin was coming from both tryptophan and tyrosine residues, whereas 293 nm wavelength excited tryptophan residues only [38].



(a)



(b)

Figure 4. *Fluorescence emission spectra in the absence and presence of vitamin C (a=free (HSA (a), BSA (b), b=1mg.ml^{-1}, c=2mg.ml^{-1}, d=5mg.ml^{-1}, e=10mg.ml^{-1}, f=20mg.ml^{-1}, g=40mg.ml^{-1}).*

Figure 4 represents the fluorescence spectra of HSA and

BSA in combination with different concentrations of vitamin C. It was observed that fluorescence spectrum exhibiting the peak maximum at 460 nm for HSA-vitamin C complexes (Figure 4a) while the peak maximum appears to be at 440 nm for BSA-vitamin C complexes (Figure 4b). The fluorescence intensity decreases with increasing concentrations of vitamin C. This indicated that there was some alteration in the microenvironment of the fluorophore Trp-213 upon interaction of both proteins with vitamin C.

Fluorescence quenching is usually classified into two types: dynamic quenching and static quenching. It can be distinguished by their different dependence on temperature and excited-state lifetime [39, 40]. For the dynamic quenching, higher temperatures will result in faster diffusion and larger amounts of collisional quenching. Therefore the quenching constant values will go up with the increase in temperature, but the reversed effect will be observed for static quenching [41]. To analyze the fluorescence quenching mechanism, the Stern–Volmer equation [42] was used:

$$\frac{F_0}{F} = 1 + K_{sv}[L] = 1 + k_q \tau_0 [L] \qquad (2)$$

where F_0 and F are protein (HSA and BSA) fluorescence intensities in the absence and presence of quencher (Vitamins C); k_q is the protein bimolecular quenching rate constant; τ_0 is the average fluorescence lifetime of protein molecule without quencher (of the order of 10^{-8} [43]); [L] is the concentration of quencher, k_{sv} is Stern-Volmer fluorescence quenching constant.

As presented in Figure 5, the Stern-Volmer plot of both complexes fluorescence intensities. The two plots showed good linear relationship, suggesting the existence of a single type of quenching (dynamic or static) and/or a single binding site. The values of the fluorescence quenching constant determined applying Eq. (2) are equal to (9.46×10^3, 4.78×10^3) L mol^{-1} for HSA/BSA-vitamin C respectively, both values are much lower than other k_{sv} values for the similar systems signalized earlier in literature [9, 44]. The values of kq which are equal to (9.46×10^{11}, 4.78×10^{11}) L mol^{-1} s^{-1} for HSA/BSA-vitamin C respectively. These values confirms clearly the existence of static (diffusion- independent) mechanism of fluorescence quenching [8, 17, 45].

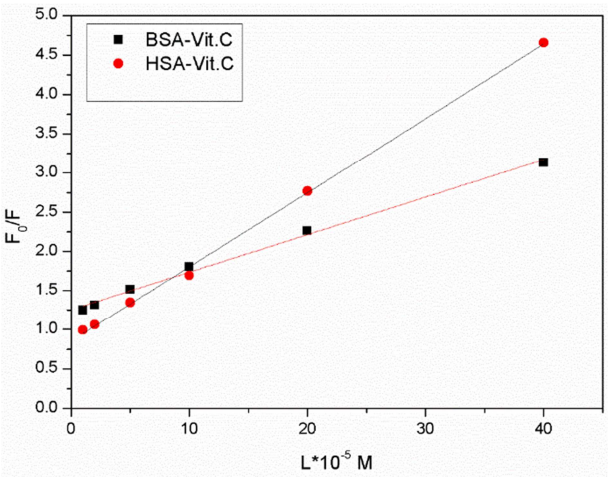


Figure 5. *The Stern-Volmer plot for HSA/BSA- vitamin C complexes.*

The calculated values of kq were greater than of the maximum dynamic quenching constant for various quencher with biopolymer ($2x10^{10}$ L.mol^{-1}.s^{-1}) [46], this suggested that the fluorescence quenching was not the result of dynamic quenching, but the consequence of static quenching [47]. When static quenching is dominant, the modified Stern-Volmer equation could be used [48].

$$\frac{1}{F_0 - F} = \frac{1}{F_0 K(L)} + \frac{1}{F_0} \qquad (3)$$

where K is the binding constant, a plot of $\frac{1}{F0-F}$ vs $\frac{1}{L}$ is presented in Figure 6. The two plots are linear and have a slope of $\frac{1}{F0\,K}$ and intercept $\frac{1}{F0}$ according to eq. (3). The values of K were found to be (1.3×10^4 M^{-1}, 1.6×10^4 M^{-1}) for HSA/BSA-vitamin C respectively, which agrees well with the values obtained earlier by UV spectroscopy and supports the effective role of static quenching.

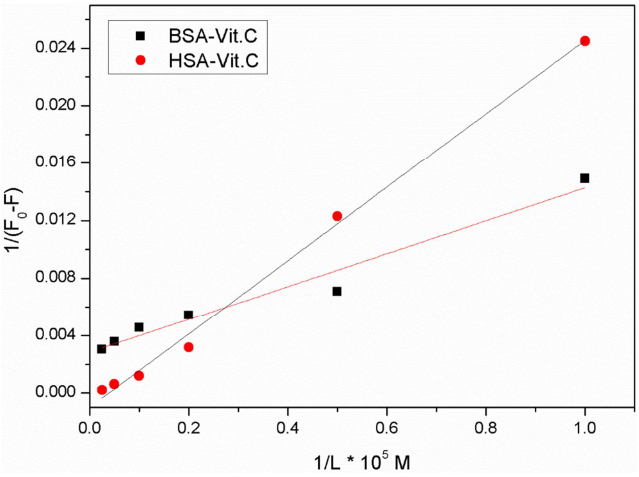


Figure 6. *The plot of $1/(F_0-F)$ vs. $1/L\times 10^5$ for HSA/BSA-Vitamin C complexes.*

4. Conclusions

One of the crucial steps in developing a new and effective drug is studying and understanding its ability to bind to albumins, as serum albumin play a key role in the transport, distribution, metabolism and excretion of drugs molecules. In this work, the interaction between HSA/BSA-vitamin C was investigated by fluorescence and UV spectroscopic techniques. The results showed that the fluorescence of HSA and BSA would be quenched with the addition of vitamin C. This change was via static quenching. The calculated binding constants of the interaction of vitamin C with both proteins using both techniques showed a good agreement but the values were found to be relatively week.

The conclusions are important in the field of pharmacology and biochemistry and are helpful for understanding the effect of vitamin C on protein function during the blood transportation process. The clear and quantitative information on the nature of this study may provide some information for its rational use in clinical practice.

Acknowledgements

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